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Specification and Drawings as originally filed, with Application for Patent Serial No: **2,391,118,** on June 21, 2002, by **UNIVERSITÉ DU QUÉBEC À MONTRÉAL,** assignee of Joanne Paquin, Jolanta Gutkowska, Bogdan A. Danalache and Marek Jankowski, for "Oxytocin as Cardiomyogenesis Inducer and Uses Thereof".

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OXYTOCIN AS CARDIOMYOGENESIS INDUCER AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the field of cell differentiation. More particularly, the present invention relates to the use of oxytocin (OT) as a cell differentiating agent, and even more specifically as an inducer of cardiomyogenesis. The present invention further relates to the use of cardiomyocytes obtained by oxytocin-induced differentiation of stem cells in the treatment of diseases associated with loss of cardiomyocytes, such as cardiac congenital malformations and aging-related heart pathologies.

BACKGROUND OF THE INVENTION

Each year, up to 7% of the three millions new-born babies in the USA have birth defects, and defects predominantly affect the heart. Furthermore, it is a well known fact that cardiovascular diseases are largely present in aging populations. There is panoply of drugs to treat such diseases or prevent their progress. Some drugs are used to improve the cardiodynamic properties of the heart (e.g. agonists/antagonists of adrenergic receptors), while others are used to reduce prejudicing conditions (e.g. substances that attenuate hypercholesterolemia). In some cases, the cardiovascular diseases are treated by surgical interventions.

Today, new prospective therapies envisage myocardial regeneration as an alternative for treating cardiovascular diseases because heart infarction, congestive heart failure and acute myocardial ischemia lead to an irreversible death of cardiac tissue (cardiomyocytes and vascular structures) which becomes replaced by scar tissue. Cardiac cell transplantation or *in situ* (trans)differentiation of non-cardiac cells into cardiomyocytes are now being considered as means to provide healthy cells to the damaged areas in order to replace the necrotized tissue and recover a sufficient number of functional cells. There is no established cardiac regenerative therapy but research for developing this kind of intervention is being pursued.

Recently, Oxytocin (OT) has been shown to have an influence on the developing heart. Also, a new role has been suggested for OT as a growth and cellular differentiation factor. A mitogenic action of OT has also been described. OT stimulates the proliferation of thymocytes and mitotic activity in the prostate epithelium, vascular endothelium and trophoblasts. Furthermore, OT has also been reported to enhance myoepithelial cell differentiation and proliferation in the mouse mammary gland. However, it has never been demonstrated nor suggested that OT could have a cardiomyogenesis activity.

Therefore, there is a need for new prospective therapies and new drugs to prevent and treat heart-related diseases, and more particularly methods and compositions wherein OT is used for inducing and/or promoting differentiation of cells and more particularly stem/progenitor cells into cardiac cells.

SUMMARY OF THE INVENTION

The present invention pertains to the use of oxytocin (OT), its gene construct and/or functional derivatives thereof as a cell differentiating agent and in compositions useful for treating or preventing diseases, such as heart diseases and in particular those associated with loss of cardiomyocytes. More particularly, the present invention pertains to the use of oxytocin, its gene construct and/or their functional derivatives thereof as an inducer of cardiomyogenesis, and more specifically as an inducer that promotes heart regeneration via the differentiation of stem/progenitor cells *in situ*. The present invention also pertains to the use of oxytocin and functional derivatives thereof to induce cardiac differentiation of stem/progenitor cell in cell culture in order to provide material for cell or tissue grafting in the heart.

According to a first aspect, the invention provides a pharmaceutical composition which comprises oxytocin and/or of a functional derivative of oxytocin in an amount effective to promote and/or induce differentiation of stem/progenitor cells into cardiac cells, and a suitable pharmaceutical acceptable diluent or carrier.

According to another aspect of the invention, oxytocin and/or its functional

derivatives, are used as an active agent in the preparation of a medication for preventing or treating a heart disease or for treating an injury to cardiac tissues. The invention also provides methods for preventing or treating a heart disease or for treating an injury to cardiac tissues, comprising the administration to a patient in need thereof of a therapeutically effective amount of oxytocin or of a functional derivative of oxytocin or the administration of a therapeutically effective amount of a composition as defined hereinabove.

According to a further aspect, the invention provides a method for inducing and/or promoting differentiation of cells and more particularly stem/progenitor cells cultured *in vitro* into cardiac cells, such as cardiomyocytes. In a preferred embodiment, the method comprises the step of providing to the *in vitro* cultured stem/progenitor cells an effective amount of oxytocin or of a functional derivative thereof. According to another aspect, the present invention provides a method to stimulate the fusion of newly-differentiated cardiomyocytes. Furthermore, the present invention provides a method for enhancing proliferation of cells and more particularly stem/progenitor cells cultured *in vitro* which comprises the step of providing to the *in vitro* cultured stem/progenitor cells an effective amount of an oxytocin-antagonist.

According to a further aspect, the invention pertains to the use of DMSO for increasing the oxytocin binding-affinity to its receptor. This may be used for inducing and/or promoting OT-related differentiation of cells.

An advantage of the present invention is that it provides effective means for maintaining or stimulating the regeneration of cardiac cells, such as cardiomyocytes, and thereby, it permits the treatment of injuries to the heart tissues. Another advantage of the present invention is that it improves the efficiency of methods for culturing cardiac cells *in vitro* either as model system or graft material.

Other objects and advantages of the present invention will be apparent upon reading the following non-restrictive description of several preferred embodiments made with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the time schedule of the differentiation of P19 cells to cardiomyocytes. P19 cells were cultivated as aggregates from day 0 to day 4 in the presence of DMSO (0.5% w/v) or oxytocin (OT) (10^{-7} M) as the agent inducing cellular differentiation. At day 4, aggregates (embryoid bodies) were transferred to tissue culture dishes or multiwell plates and grown in the absence of the agent. Micrographs (100X magnification) show undifferentiated cells and day 14 cardiomyocyte derivatives obtained after DMSO or OT treatment.

Figures 2A and 2B show that oxytocin (OT) induces myocyte immunological markers in P19 cells. P19 cell aggregates were treated from day 0 to day 4 with DMSO, OT or no differentiation agent, and stained on day 14 with anti-MHC or anti-DHPR-alpha1 antibodies. **Figure 2A** are micrographs (100X magnification) showing day 14 cells that were exposed to OT treatment. Normal light and fluorescence pictures are presented side by side. **Figure 2B** is a graph showing immunoreactivity (ir) signals obtained for undifferentiated cells grown in monolayers (Undiff.), non-treated cell aggregates (No inducer) and cell aggregates treated with DMSO or OT. Immunoreactive foci were absent (0), very rare (slightly above zero), or abundant (++ and +++). Results are representative of 3 independent experiments. Although not presented, aggregates were also treated for 6 days with OT. There was no difference with the 4-day treatment.

Figures 3A, 3B and 3C show comparison of the cardiomyogenic effect of oxytocin (OT) and DMSO. **Figure 3A** shows the retention of rhodamine^{123} in non-induced and induced P19 cultures. P19 cells were cultured as aggregates for 4 days in the absence (No inducer) or the presence of OT or DMSO, using 1 petri dish per treatment. At day 4, aggregates of each petri dish were evenly distributed in wells of a 24-well tissue culture plate. At day 8, the cells were incubated for 45 min in the presence of 1 µg/ml of the dye, washed extensively, and cultured in complete medium without dye for 48 h. The photograph shows rhodamine^{123} retention by cells induced by OT at day 10 of culture. The retained dye was fluorimetrically quantified

for each well, and the results are reported as the means ± SEM of 24 determinations. The symbol * indicates a highly significant difference with No inducer, and symbol # , a highly significant difference between OT and DMSO treatments ($p < 0.001$). **Figure 3B** is a graph showing the time course of appearance of beating cell colonies upon treatment with different agents. Aggregates of 1 petri dish treated for 4 days with the indicated agent(s) were evenly distributed in wells of a 24-well tissue culture plate. Then, each plate was examined at 2-day intervals for the number of wells containing beating cell colonies. The results are representative of 3 independent differentiation experiments. **Figure 3C** shows the RT-PCR analysis of ANP gene transcript in undifferentiated and induced cultures. Cell aggregates were exposed to OT or DMSO in the absence or presence of OTA from day 0 to day 4, and RNA was extracted at day 14 of the differentiation protocol. ANP transcript was also evaluated in undifferentiated cells grown in monolayers (Undiff.). Mouse heart ventricle mRNA was used as a positive control. Levels of ANP mRNA were adjusted by dividing by corresponding GAPDH mRNA and then expressed as the percentage of the Undiff. value. Results are reported as the means ± SEM of 5 independent studies. The symbol * indicates a significant difference with Undiff., and symbol §, a significant difference between OT and OT + OTA treatments ($p < 0.05$).

Figures 4A, 4B, and 4C show that OT and DMSO increase OTR expression in P19 cells. P19 cells were cultured as aggregates for 4 days in the absence (No inducer) or presence of DMSO (0.5%), OT (10^{-7} M) and/or OTA (10^{-7} M), and then plated in tissue culture dishes where they grew in the absence of the agent. At day 14 of differentiation, the cells were examined for OTR expression, together with undifferentiated (Undiff.) cells grown in monolayers. The results are representative of 3 independent differentiation experiments. **Figure 4A** are micrographs showing the immunocytochemistry results. **Figure 4B** shows the immunoblotting results (20 µg protein/lane). **Figure 4C** shows the RT-PCR analysis.

DETAILED SCRIPTION OF THE INVENTION

The present invention generally pertains to the use of oxytocin, its gene construct and/or their functional derivatives thereof as a cell differentiating agent in compositions useful for treating or preventing diseases, such as heart diseases and in particular those associated with loss of cardiomyocytes. More particularly, the present invention pertains to the use of oxytocin, its gene construct and/or their functional derivatives thereof as an inducer of cardiomyogenesis, and more specifically as an inducer that promotes heart regeneration via the differentiation of stem/progenitor cells *in situ*. The present invention also pertains to the use of oxytocin or its functional derivatives thereof to induce cardiac differentiation of stem/progenitor cell in cell culture in order to provide material for cell or tissue grafting in the heart. As used herein, the term "stem/progenitor cell" refers to any stem/progenitor cell having the capacity of being differentiated into cardiomyocytes. Preferred stem/progenitor cells contemplated by the present invention are embryonic stem cells, or stem cells of developed tissues which the cell phenotype is known but are still capable of transdifferentiation, i.e. to differentiate to another cell phenotype.

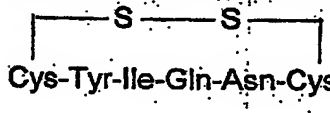
The present invention thus provides a novel cell differentiating agent and more particularly a new cardiomyogenic factor. As used herein, "cardiomyogenic factor" refers to any compound (or to any mixture of compounds) that promotes the genesis, maturation, growth, and regeneration of cardiac cells, and more specifically promotes stem/progenitor cells differentiation into cardiomyocytes.

More particularly, the present invention describes the use of oxytocin in a pharmaceutical composition and in a method for promoting the genesis, maturation, growth, and regeneration of cardiac cells. The cardiac cells that are most susceptible to benefit from the composition of the invention are newly differentiated cardiomyocytes. Also, the present invention relates to the use of oxytocin for the preparation of a composition or a medicament for the treatment or prevention of diseases, such as heart diseases and in particular those associated with loss of cardiomyocytes. Furthermore, the present invention relates to the use of DMSO for increasing the oxytocin binding-affinity to its receptor.

The pharmaceutical composition of the invention thus comprises oxytocin

and/or of a functional derivative of oxytocin in an amount effective to promote and/or induce differentiation of stem/progenitor cells into cardiac cells, and a suitable pharmaceutical acceptable diluent or carrier. Preferably, the composition of the present invention comprises DMSO for increasing the oxytocin binding-affinity to its cell receptor.

Oxytocin is a nonapeptide with two cysteine residues that form a disulfide bridge between positions 1 and 6 and corresponds to the formula:



Thus, the oxytocin and functional derivatives thereof according to the present invention are preferably substantially pure oxytocin produced by chemical synthesis, or purified from plasma and various tissues, but preferably from the pituitary gland, or produced by recombinant techniques. As generally understood and used herein, the term substantially pure refers to an oxytocin preparation that is generally lacking cellular or other undesirable components.

A "functional derivative", as is generally understood and used herein, refers to a protein sequence that possesses a functional biological activity that is substantially similar to the biological activity of the whole protein sequence. A functional derivative of a protein may or may not contain post-translational modifications such as covalently linked carbohydrate, if such modification is not necessary for the performance of a specific function. The term "functional derivative" is intended to the "fragments", "segments", "variants", "analogs" or "chemical derivatives" of a protein.

The terms "fragment" and "segment" as are generally understood and used herein, refer to a section of a protein, and are meant to refer to any portion of the amino acid sequence.

The term "variant" as is generally understood and used herein, refers to a protein that is substantially similar in structure and biological activity to either the protein or fragment thereof. Thus two proteins are considered variants if they possess a common activity and may substitute each other, even if the amino acid sequence, the secondary, tertiary, or quaternary structure of one of the proteins is

not identical to that found in the other.

The term "analog" as is generally understood and used herein, refers to a protein that is substantially similar in function to oxytocin. Preferred OT analogs include for instance extended forms of OT such as OT-Gly, OT-Gly-Lys and OT-Gly-Lys-Arg. These extended forms are biological oxytocin precursors in vivo.

As used herein, a protein is said to be a "chemical derivative" of another protein when it contains additional chemical moieties not normally part of the protein, said moieties being added by using techniques well known in the art. Such moieties may improve the protein solubility, absorption, bioavailability, biological half life, and the like. Any undesirable toxicity and side effects of the protein may be attenuated and even eliminated by using such moieties. For example, OT and OT fragments can be covalently coupled to biocompatible polymers (polyvinyl-alcohol, polyethylene-glycol, etc) in order to improve stability or to decrease antigenicity.

The amount of oxytocin and/or functional derivatives thereof present in the composition of the present invention is a therapeutically effective amount. A therapeutically effective amount of oxytocin is that amount of oxytocin or derivative thereof necessary so that the protein acts as a cardiomyogenic factor, and more particularly the amount necessary so that the protein promote the maturation, growth, and regeneration of cardiac cells, and more specifically, cardiomyocytes. The exact amount of oxytocin and/or functional derivatives thereof to be used will vary according to factors such as the protein biological activity, the type of condition being treated as well as the other ingredients in the composition. Typically, the amount of oxytocin should vary from about 10^{-15} M to about 10^{-2} M. In a preferred embodiment, oxytocin is present in the composition in an amount from about 10^{-10} M to about 10^{-4} M, preferably from about 10^{-9} M to about 10^{-6} M. In the preferred embodiment, the composition comprises about 10^{-7} M of oxytocin.

Further therapeutic agents can be added to the composition of the invention. For instance, the composition of the invention may also comprise therapeutic agents such as modulators of the cardiodynamic properties of the heart (agonists/antagonists of adrenergic receptors, activators of neurohormones, cytokines, signaling second messengers such as cAMP / cGMP / calcium or their analogs, inhibitors of the degradation of second messengers), growth factors, steroid

/ glucocorticoid / retinoid / thyroid hormones which modulate heart gene expression, proteases / protease inhibitors / cell adhesion proteins / angiogenic factors that modulate cardiac tissue organization and/or vascularization, antioxidants that provide cell protection to endogenous cardiac tissue as well as to exogenous cardiomyocyte cultures before, during and after engrafting, anticoagulants, immunosuppressive drugs.

Further to the therapeutic agents, the pharmaceutical compositions of the invention may also contain metal chelators (proteinic or not), metal scavengers (proteinic or not), coating agents, preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts, buffers, coating agents and/or antioxidants. For preparing such pharmaceutical compositions, methods well known in the art may be used.

The method of preparation of the composition of the invention consists simply in the mixing of purified oxytocin and other component(s) in a suitable solution in order to get a homogenous physiological suspension. A suitable solution is an isotonic buffered saline solution comprising sodium, potassium, magnesium or manganese, and calcium ions at physiological concentrations, that is it mimics the ion composition of the extracellular fluid. The solution has an osmotic pressure varying from 280 to 340 mOsmol, and a pH varying from 7.0 to 7.4. The buffered saline solution can be selected from the group consisting of Krebs-Henseleit 's, Krebs-Ringer's or Hank's buffer, as examples. Cholesterol can be also added since it may help to the high affinity binding of oxytocin to its receptor.

The composition of the invention could be suitable to treat and/or prevent diseases such as cardiovascular diseases or treat an injury to heart tissues. Cardiovascular diseases which could be treated include cardiac congenital malformations (*e.g.* cardiac atrophy, cardiac hypertrophy, defective cardiac chamber organization) or dysfunctions that could be caused by stress conditions during the fetal life or at birth, including ischemic conditions, infections by microorganisms, exposure to teratogenic toxicants, substances or drugs. Cardiovascular diseases which could be treated also include aging-related heart pathologies, such as heart infarction, congestive heart failure, and acute myocardial ischemia.

The composition could also be involved in the modulating heart development during embryogenesis by inducing cardiomyogenesis. The composition of the invention may thus be administered during gestation to correct development of the heart.

The composition of the invention may be administered alone or as part of a more complex pharmaceutical composition according the desired use and route of administration. For instance, the composition of the invention could comprise a vector, such as a plasmid or a virus, comprising a DNA sequence coding for native oxytocin, coding for a modified/fusion oxytocin protein having an increased cardiomyogenic activity, or an increased stability. Anyhow, for preparing such compositions, methods well known in the art may be used.

Oxytocin and/or its derivatives may be coupled to a biocompatible polymer (e.g. polyethylene glycol, polyvinyl alcohol) to reduce antigenicity when administered parenterally.

The composition of the invention and/or more complex pharmaceutical compositions comprising the same may be given via various routes of administration. For instance, the composition may be administered in the form of sterile injectable preparations, for example, as sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparations may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents. They may be given parenterally, for example intravenously, intramuscularly or sub-cutaneously by injection or by infusion. The composition may also be administered *per os* (*e.g.* capsules), nasal spray, transdermal delivery (*e.g.* iontophoresis). Suitable dosages will vary, depending upon factors such as the amount of each of the components in the composition, the desired effect (fast or long term), the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated.

Even more preferably, the composition of the invention and/or more complex pharmaceutical compositions comprising the same may be given by direct injection into the heart at the site of infarction or injury. Indeed, damaged sites were shown

to attract newly added cardiomyocytes or progenitor cells.

Oxytocin or a functional derivative thereof could also be used in methods for culturing cardiac cells *in vitro*. By providing an effective amount of oxytocin to *in vitro* cultured stem/progenitor cells, it will induce the differentiation of the cultured stem/progenitor cells into cardiac cells, such as cardiomyocytes, and then will promote the aggregation of cardiac cells and promote the tissular organization of *in vitro* cultured heart tissues. Oxytocin or a functional derivative thereof could thus be very useful for providing cardiac tissues for transplant purposes.

Therefore, a related aspect of the invention relates to a method for inducing cardiomyogenic differentiation from cells, such as stem cells. In a preferred embodiment, the method comprises the step of contacting the stem cells with an effective amount of oxytocin. According to another aspect, the present invention provides a method to stimulate the fusion of newly-differentiated cardiomyocytes. The cells are contacted with about 10^{-10} M to about 10^{-4} M of OT, preferably from about 10^{-9} M to about 10^{-6} M of OT, for about 8 h to about 14 days.

The present invention further provides a method for enhancing proliferation of cells and more particularly stem/progenitor cells cultured *in vitro* which comprises the step of providing to the *in vitro* cultured stem/progenitor cells an effective amount of an oxytocin-antagonist. Preferred oxytocin-antagonists are those that enhance cell proliferation, by preferably blocking processes that initiate or maintain the differentiated state of the cell. An example of a suitable oxytocin-antagonist is $[d(CH_2)_5^1, Tyr(Me)^2, Thr^4, Orn^8, Tyr\text{-}NH_2^9]$-vasotocin (OTA).

It will be understood by one skilled in the art that the methods and compositions contemplated by the present invention when applicable, may advantageously be used either *in vitro*, *ex vivo* and/or *in vivo*.

EXAMPLE

The following example is illustrative of the wide range of applicability of the present invention and is not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any method and material similar or equivalent to those described herein

may be used in practice for testing of the present invention, the preferred methods and materials are described.

Introduction

Oxytocin (OT), a nonapeptide largely expressed in the hypothalamus, has long been recognized as a female reproductive hormone necessary for uterine contraction during parturition, timing and amplification of labour, milk ejection during lactation, and ovulation (1). However, the last decades have shed new light on OT functions. It has been shown that both sexes have equivalent concentrations of OT in the hypophysis and plasma as well as a similar number of oxytocinergic neurons in the hypothalamus (2), and respond to the same stimuli for OT release (3, 4). It also appears that reproductive functions and maternal behaviour are preserved in $OT^{-/-}$ mutant mice (5). Both $OT^{-/-}$ males and females are fertile, and females are capable of parturition although they lack the milk ejection reflex (5, 6). These observations indicate that OT is not essential for reproduction, and data now underline the involvement of OT in sexual behaviour, cognition, memory, tolerance, adaptation, food and water intake, and cardiovascular functions (1, 7, 8).

Recently, a new role has been suggested for OT as a growth and cellular differentiation factor. The antiproliferative effect of OT, mediated by OT receptors (OTR), has been documented in breast cancer cells (9) and other tumors (10-12). In contrast to its effect on tumoral cells, a mitogenic action of OT has also been described. OT stimulates the proliferation of thymocytes (13, 14) and mitotic activity in the prostate epithelium (15), vascular endothelium (16) and trophoblasts (17). OT has also been reported to enhance myoepithelial cell differentiation and proliferation in the mouse mammary gland (18). The possibility that OT has trophic effects on the embryo has not been investigated intensively. However, OT has been shown to have an influence on the developing heart: OT administered in excess to the fetus may impair cardiac growth in humans and rats (19, 20), and OTR suppression by specific OT antagonists (OTA) in the early stage of chicken egg development leads to cardiac malformation in the embryos (21). It is not known whether the trophic effects of OT on the heart are direct or indirect.

OT's indirect actions could be related to its cardiovascular functions observed in adult rats (7, 22-24). Indeed, we uncovered the entire OT/OTR system in the rat heart, and showed that cardiac OTR activation is coupled to the release of atrial natriuretic peptide (ANP), a potent diuretic, natriuretic and vasorelaxant hormone that is also involved in cell growth regulation (7, 8). A role for ANP in cardiomyogenesis has even been suggested by Cameron et al. (25). In support of a potential action of OT on cardiac development, a maximal OT protein level was seen in the heart at day 21 of gestation and postnatal days 1-4, when cardiac myocytes are at a stage of intense hyperplasia (26).

The P19 mouse embryonal carcinoma cell line is an established model of cell differentiation. Developmentally, pluripotent P19 cells give rise to the formation of cell derivatives of all 3 germ layers (27) (28) and appear to differentiate via the same mechanisms as normal embryonic stem cells (27, 29). When cultured in the presence of 10^{-6} M retinoic acid (RA), a physiologically-relevant morphogen, P19 cells efficiently (≥ 95%) differentiate to neurons (27, 30, 31). The solvent DMSO induces cardiac differentiation, albeit not as efficiently (≤ 15%) (27, 32). DMSO has been shown to activate essential cardiogenic transcription factors, such as GATA-4 and Nkx-2.5 (32, 33). However, the mechanisms responsible for triggering these genes in the embryo are still unknown, as is the mode of action of DMSO with respect to the cardiomyogenic program in P19 cells.

In the present example, the inventors investigated whether OT induces differentiation of P19 cells into a cardiomyocyte phenotype. The results confirm that OT has a potential naturally-occurring cardiomorphogen activity.

Materials And Methods

Culture and differentiation of P19 cells

P19 cells were propagated and differentiated according to the procedures of Rudnicki and McBurney (28), with minor modifications. Undifferentiated cells were propagated in complete medium containing α-modified Eagle's minimal essential medium (GIBCO-BRL Burlington, Ontario, Canada) supplemented with 2.5% heat-inactivated fetal bovine serum, 7.5% heat-inactivated donor bovine serum (Cansera

International, Rexdale, Ontario, Canada), and the antibiotics (GIBCO-BRL) penicillin G (50 U/ml) and streptomycin (50 µg/ml). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 2 days. The general protocol used for differentiation of P19 cells is depicted in Figure 1.

Differentiation was routinely induced with DMSO. Briefly, 0.25×10^6 cells were allowed to aggregate for 4 days in non-adhesive bacteriological grade petri dishes (6-cm diameter) containing 5 ml complete medium, in the presence of 0.5% (v/v) DMSO (Sigma Chemical Co., St. Louis, MO). At day 2 of aggregation, the inducing culture medium was replenished. At day 4, aggregates were transferred to tissue culture grade vessels (10-cm diameter dishes or 24/48-well plates), and cultured in complete medium in the absence of differentiation-inducing agent. Aggregation was also done in the absence of DMSO, and in the presence of 10^{-7} M OT and/or 10^{-7} OTA ([d(CH_2)$_5$^{1},Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH_2^{9}]-vasotocin), both from Peninsula Laboratories Inc. (San Carlos, CA). The cell populations were analyzed at days 10-14 of the entire differentiation protocol, at a time cardiac cells normally beat synchronously.

Cell morphology, staining and immunocytochemistry

Examinations were done under a Zeiss® inverted microscope (Zeiss IM, Carl Zeiss, Jena, Germany) equipped with phase-contrast objectives, filters for rhodamine and fluorescein fluorescence, a MC 100® camera and a photoautomat unit. Micrographs were taken with Kodak Technical Pan® film (for cell morphology) or with Kodak T-Max 400® or Elite-II 100® film (for fluorescence).

For morphological examination, cells were grown directly onto the plastic surface of tissue culture vessels. For staining with rhodamine^{123} (Sigma), day-4 aggregates were distributed in 24-well culture plates and grown until day 8. Then, dye was added to the culture medium at a final concentration of 1 µg/ml for 45 min, and afterwards, the cells were washed extensively with phosphate-buffered saline (PBS) and cultured for 48 h in the absence of the dye. Dye retained by cells in each well was measured by a fluorescence microplate reader (SPECTRA Max Gemini®, Molecular Devices, Sunnyvale, CA) at 505 nm for excitation and 534 nm for emission.

For immunocytofluorescence studies, cells were grown onto glass coverslips coated with 0.1% gelatin. They were then fixed by 20-min incubation in PBS containing 4% paraformaldehyde, rinsed in PBS and stored at 4°C in this buffer until used. All subsequent steps of permeabilization, washing and incubation with antibodies were performed at room temperature. Fixed cells were permeabilized for 10 min in PBS containing 0.005% saponin, blocked for 60 min in PBS-BSA-saponin (PBS containing 1% bovine serum albumin and 0.005% saponin), incubated for 45 min with the primary antibody diluted 1/50 and for 45 min with a fluorescein-conjugated swine anti-goat IgG antibody (Biosource International, Camarillo, CA) diluted 1/1000. PBS-BSA-saponin was used for washing between incubations and antibodies were diluted in the same buffer but containing 1.5% normal swine serum (Jackson Immuno Research Laboratories Inc., West Grove, PA). Coverslips were mounted in PBS containing 50% glycerol, and immediately examined under the microscope. The primary antibodies were all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and produced in goat: antibody C-20 against OT receptor (OTR), antibody K-16 against sarcomeric myosin heavy chain (MHC), and antibody N-19 against dihydropyridine receptor-alpha1 (DHPR-alpha1).

Analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted with TRIzol® Reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), and poly(A)$^+$mRNA was affinity purified from 200 µg of total RNA onto Oligotex® mRNA columns (Qiagen, Mississauga, Ontario, Canada), as per the manufacturers' instructions. First-strand cDNA was synthesized in a final volume of 40 µl containing first-strand buffer, 3 µg of cellular RNA, 4 µl of hexanucleotide primers (Amersham-Pharmacia, Baie d'Urfé, Quebec, Canada), and avian myeloblastosis virus reverse transcriptase (12 units/µg RNA; Invitrogen). First-strand cDNA (5 µl) was then used for PCR amplification with OTR, ANP or GAPDH exon-specific oligonucleotide primers in a Robocycler Gradient 40 thermocycler (Stratagene, La Jolla, CA). Sequences of mouse OTR and ANP genes have been described (26, 34). Conditions for RT-PCR analysis of mouse OTR were adapted from Wagner et al. (6, 7). For all PCR studies the number of cycles used was within the linear range of amplification. The OTR sense and antisense primers

were respectively the 22-bp 5'-AAGATGACCTTCATCATTGTTC-3' and the 23-bp 5'-CGACTCAGGACGAAGGTGGAGGA-3'. Amplification was performed over 32 cycles, each involving 1 min at 94°C, 1.5 min at 62°C and 1.5 min at 72°C, and was terminated by a 5-min final extension at 72°C. The ANP antisense and sense primers were respectively the 24-bp 5'-GTCAATCCTACCCCCGAAGCAGCT-3' and the 20-bp 5'-CAGCATGGGCTCCTTCTCCA-3'. Amplification was performed over 25-30 cycles, each involving 1 min at 94°C, 1 min at 65°C and 3 min at 72°C, and was terminated by a 5-min final extension at 72°C. The amplification of GAPDH mRNA, a constitutively and ubiquitously expressed gene, served as an internal standard for RT-PCR analysis. The 23-bp antisense primer 5'-CAGTGATGGCATCCACTGTGGTC-3' and the 23-bp sense primer 5'-AAGGTCGGTGTCAACCCATTTGGCCGT-3' were used. Amplification was performed over 23 cycles, each involving 1 min at 94°C, 1.5 min at 59°C and 2 min at 72°C.

Western blot analysis

Cells were collected by scraping, homogenized in sucrose buffer (20 mM Hepes/Tris, pH 7.4, containing 250 mM sucrose and 20 µg/ml of the protease inhibitor phenylmethylsulfonyl fluoride), then centrifuged at 3000 *g* for 10 min at 4°C to remove debris. The supernatants were centrifuged at 100 000 *g* for 45 min at 4°C, and the pellets were resuspended in sucrose buffer for analysis of protein content by a modified Bradford assay (30). Aliquots (20 µg protein) were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions (35) followed by electrotransfer onto pure nitrocellulose membrane (Hybond-C, Amersham-Pharmacia). Molecular size calibration was achieved using Broad Standard Solution (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). The nitrocellulose blots were blocked overnight with 5% nonfat milk in Tris-buffered saline (TBS: 20 mM Tris-Cl, pH 8.0, 140 mM NaCl, 1% BSA and 0.1% Tween-20), then probed with goat C20 antibody (anti-OTR; 1/1,000) for 2 h at room temperature. Antibody incubations and washes were performed in TBS throughout. Detection was realized by enhanced chemiluminescence with an Amersham-Pharmacia ECL® kit and an appropriate

peroxidase-conjugated secondary antibody (29). Autoluminograms were developed in an AFP Imaging Mini-med 190® X-Ray Film Processor (AFP Corp., Elmsford, NY).

Statistics

Results are reported as the mean values ± SEM. Comparisons between treatments were done by unpaired Student's *t* test.

Results

Using the time schedule depicted in Figure 1, treatment of P19 cell aggregates with 10^{-7} M OT induced the formation of rhythmically-beating cells resembling primary cardiomyocytes isolated from the heart of newborn animals. A similar phenotypic change was already reported for treatment with 0.5-1% DMSO (27, 28, 30, 32). We observed that aggregates treated with OT or DMSO had a 1.5-fold smaller mean diameter than their untreated counterparts (data not shown), a finding that could reflect the antimitotic activity of OT and DMSO.

We examined whether treatment of cell aggregates with OT induced the expression of the cardiac muscle markers sarcomeric MHC and DHPR-alpha1. Sarcomeric MHC is expressed in contractile muscular cells as is DHPR-alpha1, a component of intracellular junctions critical for the coupling of excitation-contraction (27, 32, 36). As presented in Figure 2B, undifferentiated cells were negative for MHC, as reported (27, 28, 32), and for DHPR-alpha1. However as with DMSO, OT induced the appearance of numerous, intense, immunoreactive foci in cell populations (Figs 2A, B). In both cases, there were cell subpopulations that did not respond positively (Fig. 2A) and seemed to be mainly undifferentiated cells according to morphological criteria. We and others have shown that undifferentiated cells remain in DMSO-treated P19 cultures by probing for Stage-specific Embryonic Antigen-1, an established marker of the undifferentiated state (27, 28, 30). Cell aggregates not exposed to OT or DMSO were not positive for MHC and DHPR-alpha1 although they sometimes showed very rare and small immunoreactive foci (Fig. 2B, No inducer). This occasional staining could be due to spontaneous differentiation events triggered by high cell densities such as those encountered in

aggregates (27, 28).

We also compared the cardiogenic potency of OT and DMSO. First, potency was simply quantitated by rhodamine^{123} retention in cells, taking advantage of the fact that this dye, which penetrates all cell types, is retained for much longer periods (days instead of hours) in cardiac cells than in other cell types (37). To meet their energy requirements for muscular contraction, cardiomyocytes have indeed abundant mitochondria, the cell organelles that accumulate rhodamine^{123}. Figure 3A shows that exposure of the cell aggregates to OT and DMSO significantly increased cellular retention of the dye by 2-3 fold compared to non-induced aggregates ($p < 0.001$), and this increase at day 10 of differentiation was even significantly higher after OT than DMSO treatment ($p < 0.001$). Since P19-derived cardiomyocytes beat in culture, we also compared the time course of appearance of beating cells after treatment of aggregates with DMSO or OT. We found that OT stimulated the production of beating cell colonies in all 24 independently growing cultures by day 8 whereas the same result was obtained in cells induced by DMSO only by day 12 (Fig. 3B). The cardiogenic action of OT was specific and receptor-mediated, since no beating cells were seen when 10^{-7} M OTA was used in place of OT or in combination with OT (Fig. 3B). Interestingly, OTA also abolished the cardiogenic action of DMSO (Fig. 3B). Finally, cardiogenic potency was evaluated via ANP expression since this peptide is abundantly produced by cardiomyocytes. The results showed that at day 14 of differentiation ANP mRNA level was significantly upregulated in OT-treated P19 aggregates as compared to undifferentiated cells ($p < 0.05$), and this upregulation was at similar level after DMSO treatment (Fig. 3C). As for cell beating, OTA prevented OT-induced upregulation of ANP expression (Fig. 3C, $p < 0.05$). Although the effect of OTA on DMSO-induced ANP expression was not statistically significant, the inhibitory tendency was observed in all experiments (Fig. 3C). The inhibitory action of OTA on DMSO cardiomyogenic properties was thus more evident by the beating than the ANP criteria. Altogether, rhodamine^{123} absorption, and the time-course formation of beating cells and abundance of ANP mRNA pointed to a potent cardiomyogenic effect of OT. In addition, the cardiomyogenic action of OT and even that of DMSO appear to involve OTR.

To further investigate the involvement of OTR in cardiomyogenesis, we examined OTR expression in P19 cells. OTR protein (Fig. 4A, B) and mRNA (Fig. 4C) were present at low levels in undifferentiated cells, indicating that these cells can respond minimally to OT. OTR expression remained at low levels in aggregates not exposed to OT or DMSO (Fig. 4C, No inducer). In contrast, intense OTR immunoreactive foci were observed in cell populations after OT or DMSO treatment (Fig. 4A). These findings corresponded to the results of Western blotting (Fig. 4B) and RT-PCR analysis of OTR (Fig. 4C), both indicating increased OTR expression. In accordance with the absence of a cardiomyogenic effect of OTA and the inhibitory action of OTA on OT-induced cardiac differentiation, OTA did not upregulate OTR expression by itself and inhibited OT-induced OTR upregulation (Fig. 4B). Thus, the OTR-dependent cardiogenic effect of OT and DMSO seems to involve upregulation of OTR expression.

Discussion

This report shows that OT added to the culture medium of P19 stem cell aggregates induced cardiomyogenic differentiation, which was demonstrated by monitoring the expression of MHC, DHPR-alpha1 and ANP cardiac markers, retention of a mitochondrial-specific dye and the appearance of beating cell colonies. The cardiogenic effect of OT was specific and mediated by OTR because it was abolished by OTA. OT also upregulated OTR expression. These results suggest a new role for the OT/OTR system in heart genesis and development.

The P19 cell line is an excellent cell differentiation model that mimics the events of early cardioembryogenesis. Differentiation of P19 cells to cardiomyocytes by aggregation and exposure to DMSO was shown to be associated with induction of the cardiac-specific subtype of endothelin receptors (38). In addition, brain natriuretic peptide and ANP were observed in newly-formed striated muscle structures upon DMSO treatment and not in undifferentiated P19 cells and their neuronal derivatives (39). In this work, DMSO- and OT-induced ANP transcript levels reached about 5-10% of that found in the adult mouse atrium - the richest site of ANP synthesis. Several transcription factors having an essential role in

cardiogenesis are upregulated in DMSO-induced P19 cells. This was shown to be the case for the zinc-finger containing GATA-4, the homeobox gene Nkx2-5, and the myocyte enhancer factor 2C (32, 33, 40), and the overexpression of either factor in P19 cells was sufficient to induce cardiac differentiation in the absence of DMSO (32, 41, 42). Little is known about the molecular mechanisms underlying the activation of these genes, but DMSO was found to increase intracellular Ca^{2+} levels and was suspected to affect a pathway that has an extracellular component, possibly serum-borne (27, 43, 44). Interestingly, our data indicate that OTR are upregulated to a similar extent by OT and DMSO, and other studies have reported that OTR function modulates intracellular Ca^{2+} concentration in some cell types (1). It is thus tempting to suggest that OT could be a serum-borne factor that is active in DMSO-induced differentiation.

One of the mechanisms by which OT and DMSO trigger cardiac differentiation involves OTR since both agents upregulated the expression of this receptor, and OTA totally abolished their cardiomyogenic action as well as prevented OT-stimulating effect on OTR expression. Homologous regulation of OTR expression by OT itself was observed in the brain and in astroglial cell cultures (46, 47). It is noteworthy that, like DMSO, RA, used at low levels (10^{-8}-10^{-9} M), induces cardiac differentiation of P19 cells (27, 28). This observation could have some relevance to the OT/OTR system since RA was shown to upregulate OT expression in the fetal heart (26).

It is believed that adult ventricular myocytes are not terminally differentiated cells and possess the capacity to proliferate in response to an injury or a hemodynamic overlead. However, the hyperplastic response of these cells (i.e. their capacity to increase the number of functional cardiomyocytes) is limited since they can undergo only a small number of divisions, and their proliferation rate may be exceeded by the rate of cell loss in damaged myocardium. The low capacity of cardiomyocytes to reactivate their proliferative program may possibly be stimulated by the presence of anti-differentiating agents. The present study would suggest that specific oxytocin antagonists, such as [d(CH_2)$_5^1$,Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH$_2^9$]-vasotocin (OTA), could be exploited to enhance cell proliferation by blocking processes that initiate or maintain the differentiated state of the cell. Indeed, OTA

was shown to abolish DMSO-induced cardiomyogenic differentiation of P19 cells. A therapeutic strategy for treatment of the injured heart could thus encompass two steps : 1) OTA administration to the organ to stimulate proliferation preferentially to differentiation, followed by 2) oxytocin administration to induce terminal differentiation into fully functional cardiomyocytes.

Several studies have proposed a role for OT as a growth and differentiation/maturation factor in a gestational/perinatal context. In the mother, OT is required for postpartum alveolar proliferation, and induces differentiation and proliferation of myoepithelial cells of the mammary gland necessary for milk ejection (1, 18). The OT/OTR system is expressed in human cumulus/luteal cells surrounding oocytes and weak OTR gene expression is even observed in oocytes (48). Moreover, when fertilized mouse oocytes are cultured with OT in vitro, they develop at a higher rate into the blastocyst stage than their unstimulated counterparts (48). Spontaneous myometrial contractures are known to occur during pregnancy in sheep and controlled contractures induced by application of OT pulses to pregnant ewes have been shown to accelerate fetal cardiovascular function (49).

All these studies thus strongly suggest involvement of the maternal and embryonal OT/OTR systems in development of the embryo, and our work points to a particular involvement of OT in the priming of cardiogenesis. We think that OT could also assist the maturation of newly-differentiated cardiomyocytes by stimulating their fusion since beating cells derived from OT-induced P19 cells formed fiber-like structures. Such a fusogenic action was recently reported for OT on skeletal myoblasts in vitro (50). Our results may find application in regenerative therapies that consider the replacement of cardiac tissue lost after injury. In this context, OT could be used as a trophic factor to assist the compensatory division of myocytes shown to occur in infarcted organs (51), or to prime the cardiomyogenesis of a variety of progenitor/stem cells to be grafted in the injured heart (52, 53).

In conclusion, our study indicates that OT primes the cardiac differentiation of embryonic stem cells, and its action is mediated by OTR and a transduction pathway(s) which has yet to be defined. These results suggest that the OT/OTR system plays an important role in heart development.

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Although preferred embodiments of the present invention have been described in detail herein and illustrated in the accompanying drawings, it is to be understood that the invention is not limited to these precise embodiments and that various changes and modifications may be effected therein without departing from the scope or spirit of the present invention.

FIGURE 1

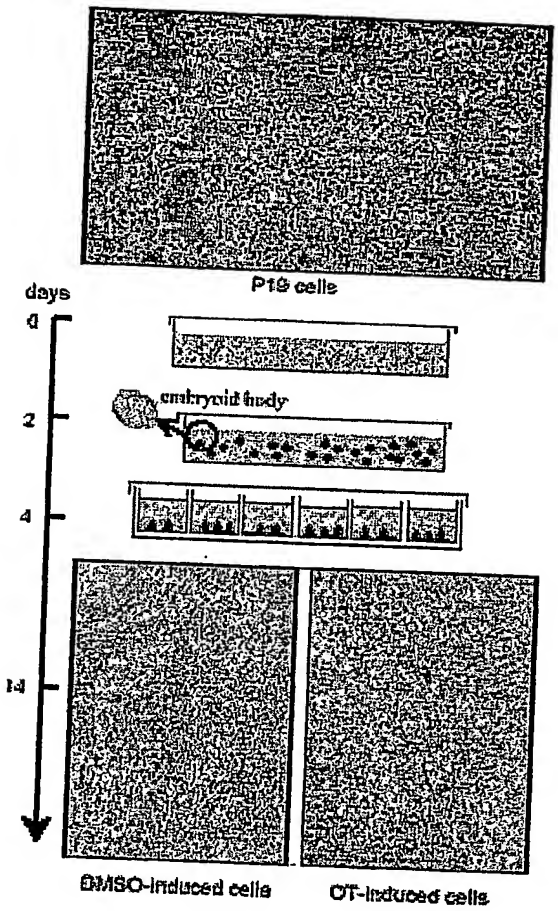

P19 cells
days
0
2
4
14
embryoid body
DMSO-induced cells
OT-induced cells

FIGURE 2

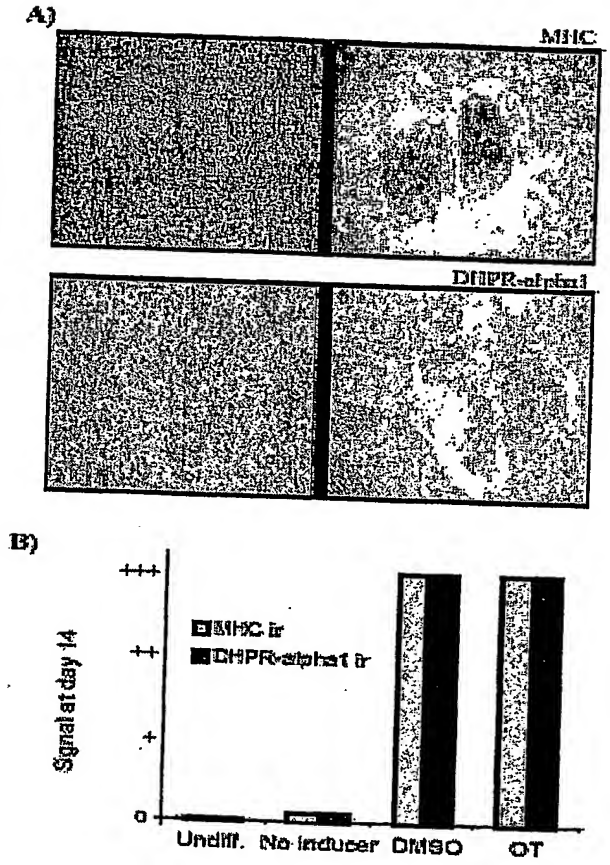

A)
MHC
DHPR-alpha1
B)
Signal at day 14
+++
++
+
0
MHC ir
DHPR-alpha1 ir
Undiff.
No inducer
DMSO
OT

FIGURE 3

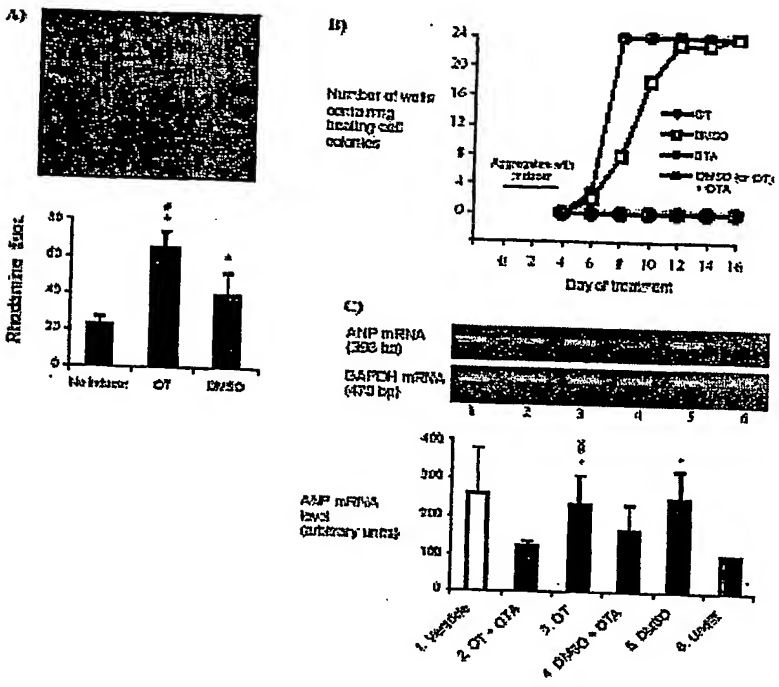
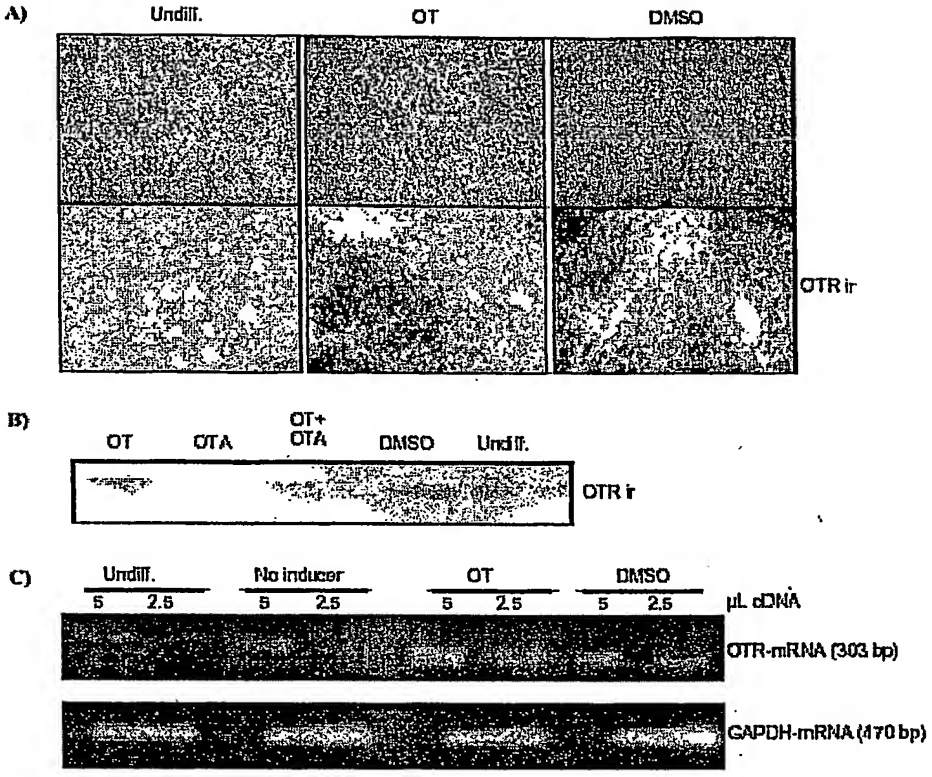

A)
Rhodamine fluor.
No inducer
OT
DMSO
B)
Number of wells containing beating cell colonies
Day of treatment
OT
DMSO
OTA
DMSO for OT + OTA
C)
ANP mRNA (393 bp)
GAPDH mRNA (479 bp)
ANP mRNA level (arbitrary units)
1. Vehicle
2. OT + OTA
3. OT
4. DMSO + OTA
5. DMSO
6. Uninduced

FIGURE 4


A)
Undiff.
OT
DMSO
OTR ir
B)
OT
OTA
OT+ OTA
DMSO
Undiff.
OTR ir
C)
Undiff.
No inducer
OT
DMSO
5
2.5
µL cDNA
OTR-mRNA (303 bp)
GAPDH-mRNA (470 bp)